EMBO reports

VE-PTP inhibition stabilizes endothelial junctions by activating FGD5

Laura J. Braun, Maren Zinnhardt, Matthias Vockel, Hannes C. Drexler, Kevin Peters, and Dietmar Vestweber

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 4th Oct 2018

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting but they also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

Given the constructive comments and support from the referees, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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REFEREE REPORTS:

Referee #1:

The manuscript by Braun et al. identifies FGD5, a RhoGEF known to activate Cdc42, as a new substrate for VE-PTP. FGD5 was of particular interest due to previous studies in this lab showing that Tie2 activation (through VE-PTP inhibition) promoted Rap1 and Rac1 activity. The authors found that FGD5 is involved in the mechanism by which inhibition of VE-PTP leads to Tie2 phosphorylation and subsequent inhibition of inflammation-induced vascular permeability. VE-PTP inhibition promoted tyrosine phosphorylation of FGD5 at Y820 through Tie2 and Rap1 activation, and induced FGD5 translocation to endothelial junctions. FGD5 acted to strengthen actin bundles

and inhibit the tension of stress fibers on endothelial junctions.

This study is novel and convincing, and is of particular interest to regulation of adherens junctional assembly. It identifies a new substrate for VE-PTP and broadens VE-PTP's function. The experiments are a logical follow-up to studies done by Frye et al. Some points in their discussion merit further experimentation such as the relationship between Rac1 and FGD5 as well as analysis of the junctional proteins involved in the endothelial stabilization seen in this study.

There are a number of protein phosphosites found in the AKB-9778-treated mouse cells that need further investigation. The role of ARHGAP12, a RhoGAP for Rac1, requires investigation due to reports implicating Rac1 in the VE-PTP/Tie2 pathway. In Frye et al., Rac1 acted downstream of Rap1 and was integral for the effects on actin and stress fiber formation as seen in this study; however, no experiments were done to address the relationship of Rac1 and FGD5. This should be done. The authors do remark on this in the discussion, however, addressing this would strengthen the paper.

The majority of the experiments done involved immunofluorescent staining and Western blotting. These data should be quantified and statistical analysis needs to be carried out. For some reason, control stains do not all look the same, suggesting high degree of variability; this is all the more reason for careful quantification, which would ensure accuracy.

VE-cadherin staining seems to be affected by the various conditions in this study. Previous studies implicate Tie2/Rap1 activation in promoting VE-cadherin adhesion. Although Frye et al. reports that VE-PTP inhibition can still stabilize endothelial junctions in the absence of VE-cadherin in vivo, this does not exclude the possibility that VE-cadherin is also involved in this mechanism. The changes in VE-cadherin staining and protein expression need to be appropriately quantified and carefully analyzed.

Staining studies of other junctional proteins found in the mass spec analysis also need to be performed. It's essential to determine changes in ZO-1 and claudin-5 in order to obtain a full picture of junctional events and strengthen the physiological relevance of the studies.

Cdc42 activity was only addressed globally. Because FGD5 junctional recruitment happens independent of FGD5 tyrosine phosphorylation, it's essential to address local changes in Cdc42 activity using a FRET biosensor.

In both Figure 2, panel E and Figure 3, panel B, there are no statistics done.

In Figure 5, panel A, the loading control is faint and unequal between all lanes. There are also some protein level changes for VE-PTP and PECAM-1. In panel D, the first two lanes do not seem different from FGD5 knockdown. Please quantify both blots and carry out a rigorous statistical analysis.

Referee #2:

VE-PTP inhibition stabilizes endothelial junctions by activating FGD5

Braun LJ, Zinnhardt M, Vockel M, Drexler HC, Peters K, Vestweber D

Multiple signaling pathways control VE-cadherin dependent vascular permeability in endothelial cells, amongst which are the VE-PTP/Tie-2 pathway, the Rap1/ArhGAP29 pathway (which dissolves radial stress fibers (RSFs)) and the Rap1/FGD5 pathway (which induces circumferential actin cables CABs)). The authors have previously found that these pathways are interconnected. In the present manuscript they reveal part of the mechanism, which beholds an intriguing triple regulation of FGD5 by VE-PTP: first, VE-PTP directly dephosphorylates FGD5 Y820. Second, VE-PTP indirectly controls the phosphorylation of FGD5, partially via Tie-2. Third, VE-PTP induces Rap1-dependent and phosphorylation-independent translocation of FGD5 to cell-cell junctions. Furthermore, functional experiments indicate that FGD5 is not only required for the formation of CABs, but also functions to decrease RSFs.

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This manuscript contains very important data that explain several open questions in the field. Furthermore, showing the mode of interplay between hitherto separate individual pathways creates some order in the meshwork of signaling pathways controlling endothelial permeability. The manuscript is clearly written in an objective manner, indicating both strong conclusions as well as disclaimers when these are appropriate. I do have a number of minor issues. If addressed properly, I recommend publication of this manuscript in EMBO Reports.

- It is concluded from figure 1B that "FGD5 could only be immunoprecipitated ... with an anti-phosphotyrosine antibody when these cells were pretreated with the VE-PTP inhibitor AKB". This conclusion seems right for this experiment, but other experiments show quite some basal phosphorylation of FGD5 (i.e. figures 1C, 8A). This is relevant for the impact of VE-PTP on the pathway: phosphorylation of Y820 is absolutely essential for Cdc42 activation (figure 9C), whereas 007 is quite potent in activating Cdc42 (figure 3E) without affecting FGD5 phosphorylation (figure 2E). This suggests that VE-PTP does not effectively impact FGD5 function. Can the authors clarify this?

- figure 2B: technically it can not be concluded from this experiment that FGD5 is phosphorylated, since it could also be a protein coprecipitating with FGD5. A nice control for this is presented in figure 8A, I suggest relocating this experiment to figure 2 for clarity. Alternatively, the experiment in figure 2B can be performed in reverse order (4G10 IP followed by detection of FGD5).

- figures 4-7: given the controversial effects of FGD5 knockdown in literature, it appears relevant to show that FGD5 knockdown does not affect the expression of FGD1 and FGD6.

- figure 4: the dextran permeability assay and ECIS assay show similar but not identical effects, which seems logical given the large size difference of the molecules that they detect. However, the authors show that basal permeability is not affected by FGD5 siRNA only using the dextran assay. The paracellular transport of ions, as detected by ECIS, might be very different between the two. This is relevant for figure 4C: if basal resistance is different upon FGD5 siRNA, than the absolute resistance drop by thrombin is different between control and FGD5 as well, obscuring potential effects of Ang1 and AKB. Indeed, thrombin appears slightly less potent in FGD5 knockdown cells in figure 7. I suggest showing the non-normalized resistances.

- figure 4C: this figure uses a different VE-PTP inhibitor than the other figures which is not mentioned anywhere in the manuscript (main text, methods). This is also incorrectly indicated in the figure legend.

- figure 5: the efficiency of the systemic siRNA is checked in lung tissue, whereas the functional assay is performed on the skin. Is knockdown efficiency similar in skin?

- figure 5: have the in vivo lysates been checked for tyrosine phosphorylation of FGD5? Are these similar to the levels observed in HUVEC?

- figure 5D: this figure is not mentioned in the text.

- figure 6: this figure basically is figure 7 without the pMLC2 staining and therefore is redundant

- figure 7: the authors correctly state that FGD5 has not been shown to affect RSFs before. However, FGD5 has been shown to bind to Radil, which dissolves RSFs. Does FGD5 phosphorylation affect Radil binding? Is this independent of Rap1, as would be expected based on the authors' findings? Does FGD5 Y820F display altered Radil binding?

- "... binding of the substrate to the active site of the phosphatase domain is largely only dependent...". Either "largely" or "only" should be removed.

- the authors restrict the functional assays with the Y820F mutant to the dextran permeability assay and Cdc42 activation assay. Has actin morphology been assayed as well? Does the mutant affect both CAB formation as well as RSF decrease? Or could it be that the mutation predominantly affects one of these. In other words: are both FGD5 functions dependent of FGD5 phosphorylation?

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Referee #3:

The manuscript reports FGD5 as a signalling target of VE-PTP and its involvement in the stabilization of endothelial junctions by VE-PTP inhibition and Tie-2 activation. Moreover, FGD5 is a direct substrate of VE-PTP and at the same time a target for Tie-2 stimulated phosphorylation. The authors also identify Y820 as the single substrate tyrosine of human FGD5 activation and show that fully activated FGD5 is required to support tension of circumferential actin bundles.

The manuscript is well written and the experiments are logically and professionally performed. The only minor thing for improvement is the Supplemental Figure 1 demonstrating the specificity of the polyclonal antibody. Full length gels with a negative control antibody (pre-bleed) should be shown.

1st Revision - authors' response 26th Feb 2019

Detailed reply to reviewers:

We thank the reviewers for their positive and constructive comments that we have addressed below as follows:

Referee #1:

The manuscript by Braun et al. identifies FGD5, a RhoGEF known to activate Cdc42, as a new substrate for VE-PTP. FGD5 was of particular interest due to previous studies in this lab showing that Tie2 activation (through VE-PTP inhibition) promoted Rap1 and Rac1 activity. The authors found that FGD5 is involved in the mechanism by which inhibition of VE-PTP leads to Tie2 phosphorylation and subsequent inhibition of inflammation-induced vascular permeability. VE-PTP inhibition promoted tyrosine phosphorylation of FGD5 at Y820 through Tie2 and Rap1 activation, and induced FGD5 translocation to endothelial junctions. FGD5 acted to strengthen actin bundles and inhibit the tension of stress fibers on endothelial junctions.

This study is novel and convincing, and is of particular interest to regulation of adherens junctional assembly. It identifies a new substrate for VE-PTP and broadens VE-PTP's function. The experiments are a logical follow-up to studies done by Frye et al. Some points in their discussion merit further experimentation such as the relationship between Rac1 and FGD5 as well as analysis of the junctional proteins involved in the endothelial stabilization seen in this study.

There are a number of protein phosphosites found in the AKB-9778-treated mouse cells that need further investigation. The role of ARHGAP12, a RhoGAP for Rac1, requires investigation due to reports implicating Rac1 in the VE-PTP/Tie2 pathway. In Frye et al., Rac1 acted downstream of Rap1 and was integral for the effects on actin and stress fiber formation as seen in this study; however, no experiments were done to address the relationship of Rac1 and FGD5. This should be done. The authors do remark on this in the discussion, however, addressing this would strengthen the paper.

We have analyzed whether our proteomic results suggesting that ArhGAP12 is a substrate of VE-PTP could be confirmed. For this, we treated HUVEC with the VE-PTP inhibitor AKB-9785 followed by immunoprecipitation of ArhGAP12 and a phosphotyrosine blot. Despite several attempts, we could not see any upregulation of phosphorylation of ArhGAP12.

In order to investigate the relationship of FGD5 and Rac1 we silenced FGD5 in HUVEC and tested whether this would block Rac1 activation by AKB-9785. Indeed, we found that Rac1 activation was completely blocked suggesting that FGD5 is upstream of Rac1. To test whether FGD5 activates Rac1 via Cdc42, we also silenced Cdc42. We found that Rac1 activation by AKB-9785 was not

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reduced by silencing Cdc42, clearly arguing that Cdc42 is not involved in FGD5 induced activation of Rac1. These results are now included in Fig. 6.

The majority of the experiments done involved immunofluorescent staining and Western blotting. These data should be quantified and statistical analysis needs to be carried out. For some reason, control stains do not all look the same, suggesting high degree of variability; this is all the more reason for careful quantification, which would ensure accuracy.

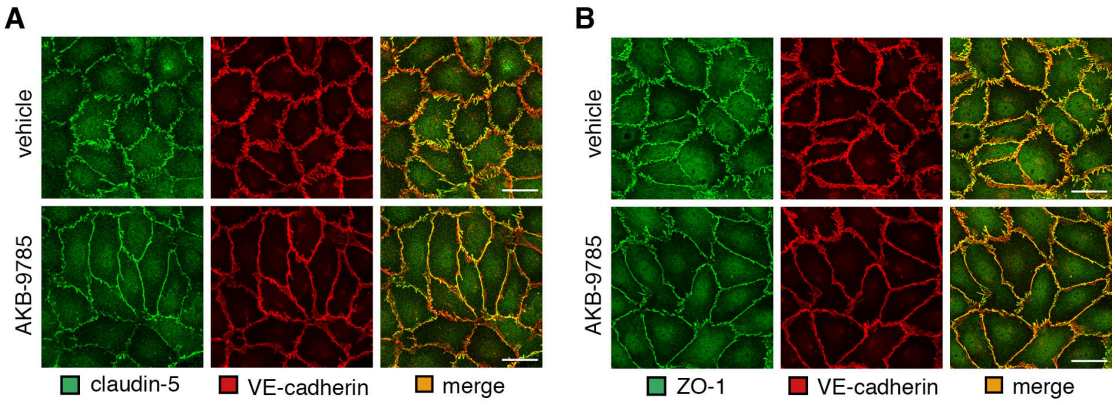
We have now quantified the staining of FGD5 at cell contacts in figure 2 (new figure 2B and F) and figure 3 (new 3E). Furthermore, we quantified the blot signals of figures 2B (new 2D), 2D (new 2G) and 3A.

VE-cadherin staining seems to be affected by the various conditions in this study. Previous studies implicate Tie2/Rap1 activation in promoting VE-cadherin adhesion. Although Frye et al. reports that VE-PTP inhibition can still stabilize endothelial junctions in the absence of VE-cadherin in vivo, this does not exclude the possibility that VE-cadherin is also involved in this mechanism. The changes in VE-cadherin staining and protein expression need to be appropriately quantified and carefully analyzed.

We have now quantified the staining of VE-cadherin at cell contacts in figure 2 (effects of 007 and AKB-9785).

Staining studies of other junctional proteins found in the mass spec analysis also need to be performed. It's essential to determine changes in ZO-1 and claudin-5 in order to obtain a full picture of junctional events and strengthen the physiological relevance of the studies.

We have treated HUVEC with vehicle and with AKB-9785 and stained the cells for claudin-5, ZO-1 and VE-cadherin. As we expected, we found that the cell contacts were straightened and focused after AKB-9785 treatment and this was seen similarly for all three junctional proteins (see below). Since it is highly likely, that the straightening of junctions is due to the effects of AKB-9785 on radial stress fibers and cortical actin bundles, we feel there is not much we can learn from these stainings for any potential effect of AKB-9785 on ZO-1 or claudin-5 function. Therefore, we prefer not to add these data to the manuscript.



1) AKB-9785 stimulation linearizes VE-cadherin, claudin-5 and ZO-1 junctional staining. *Confluent HUVEC monolayers were treated with 5 µM AKB-9785 or vehicle for 30 min, fixed, permeabilized and stained for VE-cadherin and claudin-5 (A) or ZO-1 (B). Scale bars 30 µM. Images are representative of 2 independent experiments.*

Cdc42 activity was only addressed globally. Because FGD5 junctional recruitment happens independent of FGD5 tyrosine phosphorylation, it's essential to address local changes in Cdc42 activity using a FRET biosensor.

It has been shown before by the Mochizuki lab (Ando et al., JCB 2013, 202:901-916) that Rap1 stimulation by the cAMP analogue 007 leads to the recruitment of FGD5 to cell junctions which is

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needed for Cdc42 activation to stabilize junctions and enhance circumferential actin. In addition, this study showed that 007-Rap1-FGD5 strongly stimulated Cdc42 FRET activity in the marginal regions of HUVEC cells containing the junctions. We show here, that inhibition of VE-PTP stimulates the same pathway via activation of Tie-2, namely Rap1, FGD5 junction recruitment and Cdc42 activation, which is needed for enhancing circumferential actin and stabilizing junctions. In addition, we show that VE-PTP inhibition stimulates FGD5 phosphorylation on Y820, which is needed for full activation of FGD5. This clearly shows that activation of FGD5 is a two-step process requiring junction recruitment and at the same time tyrosine phosphorylation of FGD5. However, despite this two-step mechanism, stimulation with merely 007 is able to stabilize junctions, since FGD5 has a low constitutive phosphorylation level (as we show) which allows some of the junction recruited FGD5 molecules to be phosphorylated and support junction stabilization. Only in the complete absence of this tyrosine (Y820F mutant), FGD5 junction recruitment is unable to stabilize junctions. Thus, the fact that junctional recruitment of FGD5 does not require Y820 does not mean that junctional FGD5 would not be phosphorylated. On the other hand, without Y820, junction-recruited FGD5 is unable to stabilize junctions arguing that these molecules at junctions need to be phosphorylated in order to stabilize junctions. Since Rap1 activation leads to strong Cdc42 FRET signals in the marginal regions of endothelial junctions (Ando et al., 2013), this result will also be seen if we treat the cells with the VE-PTP inhibitor, since it activates Rap1 and triggers FGD5 recruitment to junctions. Therefore, we are afraid that we may not gain much additional insight by re-doing such Cdc42 FRET experiments with another stimulus that also activates Rap1.

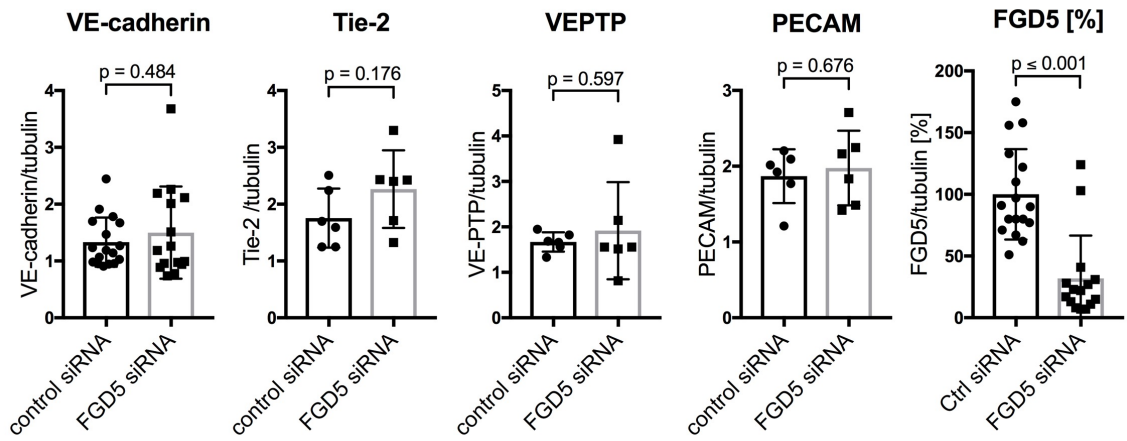
In both Figure 2, panel E and Figure 3, panel B, there are no statistics done.

We have now marked the statistical significance in figures 2H (former 2E) and 3B.

In Figure 5, panel A, the loading control is faint and unequal between all lanes. There are also some protein level changes for VE-PTP and PECAM-1. In panel D, the first two lanes do not seem different from FGD5 knockdown. Please quantify both blots and carry out a rigorous statistical analysis.

We have now quantified the blot signals for VE-cadherin, Tie-2, VE-PTP, PECAM-1 and FGD5 from all immunoblots representing all mice that were analyzed in permeability assays (see below). In fig. 5B the quantitation of the FGD5 signals was done in comparison to the VE-cadherin signals. Below all quantifications were done in comparison to the α-tubulin levels. This quantification clearly shows that the FGD5 expression level is clearly reduced, whereas there were no significant changes for the expression of the other proteins.

In addition, we have replaced the bottom panel of figure 5A and the top panel of figure 5D for a longer exposure of the same signals. This way, the signals are better comparable.



2) FGD5 knockdown in vivo does not affect the expression of VE-cadherin, Tie-2, VE-PTP or PECAM-1. *Blot signals intensities of the indicated antigens were quantified for all animals and are presented relative to signal intensities of α-tubulin signal intensities of the same sample.*

Referee #2:

VE-PTP inhibition stabilizes endothelial junctions by activating FGD5

Braun LJ, Zinnhardt M, Vockel M, Drexler HC, Peters K, Vestweber D

Multiple signaling pathways control VE-cadherin dependent vascular permeability in endothelial cells, amongst which are the VE-PTP/Tie-2 pathway, the Rap1/ArhGAP29 pathway (which dissolves radial stress fibers (RSFs)) and the Rap1/FGD5 pathway (which induces circumferential actin cables CABs)). The authors have previously found that these pathways are interconnected. In the present manuscript they reveal part of the mechanism, which beholds an intriguing triple regulation of FGD5 by VE-PTP: first, VE-PTP directly dephosphorylates FGD5 Y820. Second, VE-PTP indirectly controls the phosphorylation of FGD5, partially via Tie-2. Third, VE-PTP induces Rap1-dependent and phosphorylation-independent translocation of FGD5 to cell-cell junctions. Furthermore, functional experiments indicate that FGD5 is not only required for the formation of CABs, but also functions to decrease RSFs.

This manuscript contains very important data that explain several open questions in the field. Furthermore, showing the mode of interplay between hitherto separate individual pathways creates some order in the meshwork of signaling pathways controlling endothelial permeability. The manuscript is clearly written in an objective manner, indicating both strong conclusions as well as disclaimers when these are appropriate. I do have a number of minor issues. If addressed properly, I recommend publication of this manuscript in EMBO Reports.

- It is concluded from figure 1B that "FGD5 could only be immunoprecipitated ... with an anti-phosphotyrosine antibody when these cells were pretreated with the VE-PTP inhibitor AKB". This conclusion seems right for this experiment, but other experiments show quite some basal phosphorylation of FGD5 (i.e. figures 1C, 8A). This is relevant for the impact of VE-PTP on the pathway: phosphorylation of Y820 is absolutely essential for Cdc42 activation (figure 9C), whereas 007 is quite potent in activating Cdc42 (figure 3E) without affecting FGD5 phosphorylation (figure 2E). This suggests that VE-PTP does not effectively impact FGD5 function. Can the authors clarify this?

This point is well taken. The fact that 007 is able to activate Cdc42 without stimulating FGD5 phosphorylation indeed implies that there must be a small pool of FGD5 molecules which is phosphorylated and sufficient to mediate the 007 effects. As the reviewer correctly points out, we saw such basal phosphorylation levels in Fig. 1C and 8A. We have now replaced the blot in figure 1B by a blot of a similar experiment at a slightly longer exposure time. This shows as before the enormous difference in pY signal intensities due to the treatment with AKB-9785, however in the absence of the VE-PTP inhibitor a weak pY immunoblot signal was still detectable. Thus, under baseline conditions in HUVEC, VE-PTP prevents most of FGD5 phosphorylation but does not do so completely. Yet, inhibiting VE-PTP or activating Tie-2 strongly stimulates FGD5 tyrosine phosphorylation far beyond the baseline level, very effectively stabilizing junctions.

- figure 2B: technically it cannot be concluded from this experiment that FGD5 is phosphorylated, since it could also be a protein coprecipitating with FGD5. A nice control for this is presented in figure 8A, I suggest relocating this experiment to figure 2 for clarity. Alternatively, the experiment in figure 2B can be performed in reverse order (4G10 IP followed by detection of FGD5).

This comment is correct, technically the tyrosine phosphorylated band in figure 2B could be a protein co-precipitated with FGD5, induced in tyrosine phosphorylation by AKB-9785 and being of the same molecular weight as FGD5. However, the suggested alternative experiment (first precipitate with 4G10 then immunoblot with anti FGD5) was already shown in the original version of our manuscript in figure 1B. The purpose of figure 2B was not so much to prove that VE-PTP inhibition does indeed trigger phosphorylation of FGD5 (this is indeed better shown in figure 1B), the aim was simply to show that 007 does not trigger FGD5 tyrosine phosphorylation.

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- figures 4-7: given the controversial effects of FGD5 knockdown in literature, it appears relevant to show that FGD5 knockdown does not affect the expression of FGD1 and FGD6.

Apart from FGD5, endothelial cells have been reported to express FGD1 and FGD6. To make sure that our FGD5 silencing approach did not affect the expression of these two other GEFs in HUVEC, we transfected the cells with FGD5 siRNA or ctrl siRNA and quantified the mRNA expression levels for all three GEFs by qRT-PCR. The results are now shown in the new part C of figure 4. The expression levels of FGD1 or FGD6 were not inhibited by our FGD5 siRNA.

- figure 4: the dextran permeability assay and ECIS assay show similar but not identical effects, which seems logical given the large size difference of the molecules that they detect. However, the authors show that basal permeability is not affected by FGD5 siRNA only using the dextran assay. The paracellular transport of ions, as detected by ECIS, might be very different between the two. This is relevant for figure 4C: if basal resistance is different upon FGD5 siRNA, than the absolute resistance drop by thrombin is different between control and FGD5 as well, obscuring potential effects of Ang1 and AKB. Indeed, thrombin appears slightly less potent in FGD5 knockdown cells in figure 7. I suggest showing the non-normalized resistances.

We now show the non-normalized resistance measurements as supplemental figure 2.

- figure 4C: this figure uses a different VE-PTP inhibitor than the other figures which is not mentioned anywhere in the manuscript (main text, methods). This is also incorrectly indicated in the figure legend.

We have corrected the name of the inhibitor in figure 4D (formerly C).

- figure 5: the efficiency of the systemic siRNA is checked in lung tissue, whereas the functional assay is performed on the skin. Is knockdown efficiency similar in skin?

We had analyzed lung tissue instead of skin, since lung has a much higher relative content of endothelial cells, which allowed us to detect FGD5 levels in lysates. Similar assays were done for skin, but were unfortunately not successful, due to high background and insufficient levels of specific immunoblot signals.

- figure 5: have the in vivo lysates been checked for tyrosine phosphorylation of FGD5? Are these similar to the levels observed in HUVEC?

We have now analyzed the in vivo baseline phosphorylation levels of FGD5 and compared this with AKB-9785 treated mice (New part C of figure 1). We found that pY-levels went up in vivo by 3-3.5 fold. In comparison, for HUVEC we had an increase in FGD5 pY level of factor 10.

- figure 5D: this figure is not mentioned in the text.

Fig. 5D has now been mentioned in the text.

- figure 6: this figure basically is figure 7 without the pMLC2 staining and therefore is redundant

We have replaced the actin, VE-cadherin panels of figure 6 by the corresponding pMLC2, actin, VE-cadherin panels of former figure 7.

- figure 7: the authors correctly state that FGD5 has not been shown to affect RSFs before. However, FGD5 has been shown to bind to Radil, which dissolves RSFs. Does FGD5 phosphorylation affect Radil binding? Is this independent of Rap1, as would be expected based on the authors' findings? Does FGD5 Y820F display altered Radil binding?

These are interesting thoughts. We have tested this in transfected HEK293 cells. First, we showed that co-expression of Tie-2-Flag with FGD5-WT-EGFP leads to strong tyrosine phosphorylation of FGD5 (supplemental figure 5A). Then we co-expressed Radil-His with either FGD5-WT-EGFP or FGD5-Y820F-EGFP and co-expressed these combinations in the presence or absence of Tie-2-Flag.

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As shown in supplemental figure 5B, Radil indeed was co-precipitated with FGD5, but this interaction neither required the presence of Y820 nor the Tie-2-Flag induced tyrosine phosphorylation of FGD5.

- *"... binding of the substrate to the active site of the phosphatase domain is largely only dependent...". Either "largely" or "only" should be removed.*

was changed

- *the authors restrict the functional assays with the Y820F mutant to the dextran permeability assay and Cdc42 activation assay. Has actin morphology been assayed as well? Does the mutant affect both CAB formation as well as RSF decrease? Or could it be that the mutation predominantly affects one of these. In other words: are both FGD5 functions dependent of FGD5 phosphorylation?*

We have performed the suggested experiments and found that the FGD5-Y820F mutant could not rescue the AKB-9785 effect on RSF decrease and CAB formation in
HUVEC silenced for endogenous FGD5, whereas re-expression of WT FGD5 did rescue these effects (results now shown in the new part E of figure 8), Thus, Y820 is needed for Cdc42 activation, junction stabilization and for the effects on the actin cytoskeleton.

Referee #3:

The manuscript reports FGD5 as a signalling target of VE-PTP and its involvement in the stabilization of endothelial junctions by VE-PTP inhibition and Tie-2 activation. Moreover, FGD5 is a direct substrate of VE-PTP and at the same time a target for Tie-2 stimulated phosphorylation. The authors also identify Y820 as the single substrate tyrosine of human FGD5 activation and show that fully activated FGD5 is required to support tension of circumferential actin bundles.

The manuscript is well written and the experiments are logically and professionally performed. The only minor thing for improvement is the Supplemental Figure 1 demonstrating the specificity of the polyclonal antibody. Full length gels with a negative control antibody (pre-bleed) should be shown.

We are now showing in supplemental Fig S1. the full length of the immunoblot which documents the specificity of our anti mouse FGD5 antibodies.

2nd Editorial Decision 27th Mar 2019

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

Your manuscript was evaluated again by former referee 1 and 2 and as you will see, both are very positive about the study and support publication without further revision.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

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REFEREE REPORTS:

Referee #1:

The revision is highly responsive.

Referee #2:

I am happy with the revised manuscript

2nd Revision - authors' response 1st Apr 2019

The authors performed the requested editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dietmar Vestweber
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-47046

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We estimate the number of biological replicates using the G*Power 3.1.9.2 power analysis program with the standard significance value $\alpha = 0.05$ and a power value of $1 - \beta = 0.8$.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	see above.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not exclude any animals from experiments. For in vitro transwell permeability assays, samples were excluded when subsequent filter stainings revealed that the endothelial monolayer was not intact.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No formal randomization procedure was used for in vitro experiments. For in vivo studies, animals of the same gender, age and genetic background were chosen and randomly allocated to groups.
For animal studies, include a statement about randomization even if no randomization was used.	Animals of the same gender, age and genetic background were chosen and randomly allocated to groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Animals were randomly allocated to groups by a person different from the investigator, minimizing subjective bias. The investigator was blinded to groups (Ctrl siRNA vs FGD5 siRNA), but not to treatment (vehicle vs AKB-9785).
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigator was blinded to groups (Ctrl siRNA vs FGD5 siRNA), but not to treatment (vehicle vs AKB-9785).
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution was visually assesed based on distribution of single data points in a boxplot + mean.
Is there an estimate of variation within each group of data?	Yes. Data are represented as mean ± SEM.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We provide citations and source of antibodies in the Materials and Methods section under "Antibodies and reagents".
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293A cell were purchased from Invitrogen and routinely tested for mycoplasma contamination in our laboratory.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Female C57Bl/6 WT mice (Janvier) at an age of 8-12 weeks were used for the experiments. Mice were housed in a pathogen-free facility with food and water ad libitum and a 12h light/dark cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All procedures were performed according to the German Tierschutzgesetz and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance is confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We provide information about the deposition of our proteomics data in the Materials and Methods section under "Nano-LC-MS/MS analysis".
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Data was deposited.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA